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BENIASH, Elia; 4615 Davis Street, Skokie, IL 60076 (US).

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(74) Agents: DEKRUIF, Rodney, D. et al.; Reinhart, Boerner, Van Deuren, Norris & Rieselbach, S.C., Attn: Linda Gabriel, Docket Clerk, 1000 North Water Street, Suite 2100, Milwaukee, WI 53202-3186 (US).

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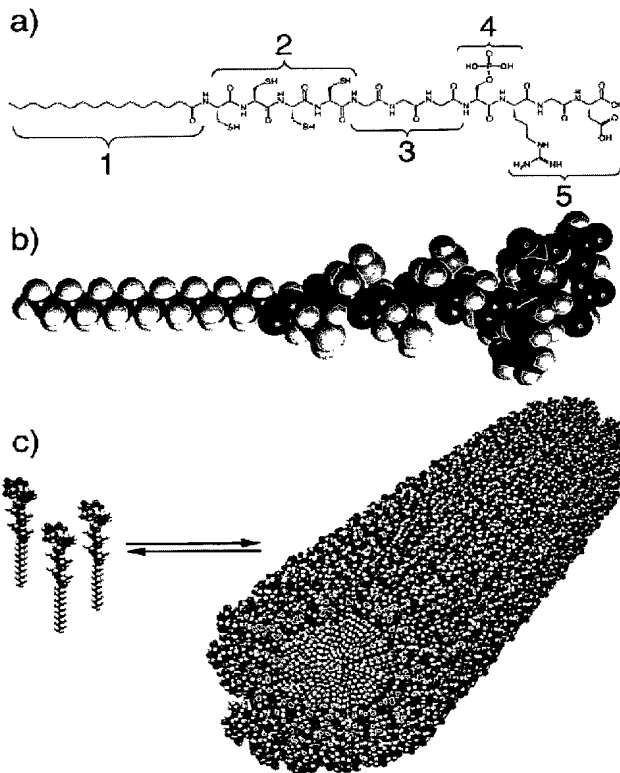
(71) Applicant: NORTHWESTERN UNIVERSITY
[US/US]; 633 Clark Street, Evanston, IL 60208 (US).

(72) Inventors: STUPP, Samuel, I.; 57 E. Delaware Place, Apt. 2802, Chicago, IL 60611 (US). HARTGERINK, Jeffrey, D.; 1115 Elmwood Avenue, Evanston, IL 60202 (US).

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(54) Title: SELF-ASSEMBLY AND MINERALIZATION OF PEPTIDE-AMPHIPHILE NANOFIBERS

(57) Abstract: Peptide-amphiphilic compositions capable of self-assembly into useful nanostructures.



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Self-Assembly and Mineralization of Peptide-Amphiphile Nanofibers

This application claims priority benefit from provisional patent application serial no. 60/333,074 filed November 14, 2001, the entirety of which is incorporated herein by reference.

The United States government has certain rights to this invention pursuant to Grant Nos. DE-FG02-00ER45810/A001, DMR9996253 and F49620-00-1-0283/P01 from, respectively, the DOE, NSF and AFOSR-MURI to Northwestern University.

Background of the Invention.

Self-assembly and biomineralization are used for fabrication of many composite materials. Bone tissue is a particularly complex example of such a composite because it contains multiple levels of hierarchical organization (S. Weiner, H. D. Wagner, *Annu. Rev. Mater. Sci.* **28**, 271-298 (1998)). At the lowest level of this hierarchy is the organization of collagen fibrils with respect to hydroxyapatite (HA) crystals. Collagen fibrils are formed by self-assembly of collagen triple helices while the HA crystals grow within these fibrils in such a way that their *c*-axes are oriented along the long axes of the fibrils (W. Traub, S. Weiner, *Proc. Nat. Acad. Sci.* **86**, 9822-9826 (1989)). The preparation of any material with structure on the nanoscale is a challenging problem. Fabrication of materials that resemble bone, even at the lowest level of hierarchical organization, is even more difficult because it involves two dissimilar organic and inorganic nanophases that have a specific spatial relationship with respect to one another. One approach, using an artificial system, has been to prepare an organic nanophase designed to exert control over crystal nucleation and growth of the inorganic component.

The controlled nucleation and growth of crystals from organic templates has been demonstrated in *in vitro* experiments and in a number of natural biomineralizing systems (S. Mann, J. P. Hannington, R. J. P. Williams, *Nature* **324**, 565-567 (1986); D. D. Archibald, S. Mann, *Nature* **364**, 430-433 (1993); S. L. Burkett, S. Mann, *Chem. Commun.* 321-322 (1996); S. I. Stupp, P. V. Braun, *Science* **277**, 1242-1248 (1997); J. Aizenberg, A. J. Black, G. M. Whitesides, *Nature* **398**, 495-498 (1999); S. R. Whaley, D. S. English, E. L. Hu, P. F. Barbara, A. M. Belcher, *Nature* **405**, 665-

668. (2000); L. Addadi, S. Weiner, *Angew. Chem., Int. Ed. Engl.* **31**, 153-169 (1992); S. Mann, *J. Chem. Soc., Dalton Tran.* 3953-3961 (1997); S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997)). These studies on templated crystal growth suggest that nucleation occurs on surfaces exposing repetitive patterns of anionic groups. Anionic groups tend to concentrate the inorganic cations creating local supersaturation followed by oriented nucleation of the crystal. Many groups have investigated the preparation of bone-like materials using three dimensional organic substrates such as poly(lactic acid), reconstituted collagen and many others, and some studies shows a similar correlation between the crystallographic orientation of hydroxyapatite when the organic scaffold is made from reconstituted collagen(G. K. Hunter, H. A. Goldberg, *Biochem. J.* **302**, 175-179 (1994); G. M. Bond, R. H. Richman, W. P. McNaughton, *J. Mater. Eng. Perform.* **4**, 334-345 (1995); J. -H. Bradt, M. Mertig, A. Teresiak, W. Pompe, *Chem. Mater.* **11**, 2694-2701 (1999); N. Ignjatovic, S. Tomic, M. Dakic, M. Miljkovic, M. Plavsic, D. Uskokovic, *Biomaterials* **20**, 809-816 (1999); F. Miyaji, H.-M. Kim, S. Handa, T. Kokubo, T. Nakamura, *Biomaterials* **20**, 913-919 (1999); H. K. Varma, Y. Yokogawa, F. F. Espinosa, Y. Kawamoto, K. Nishizawa, F. Nagata, T. Kameyama, *J. Mater. Sci.: Mater. Med.* **10**, 395-400 (1999); A. Bigi, E. Boanini, S. Panzavolta, N. Roveri, *Biomacromolecules* **1**, 752-756 (2001); M. Kikuchi, S. Itoh, S. Ichinose, K. Shinomiya, J. Tanaka, *Biomaterials* **22**, 1705-1711 (2001)). However, such results have never been demonstrated in a pre-designed and engineered self-assembling molecular system.

Brief Description of the Drawings.

Figure 1. In accordance with this invention: a) Chemical structure of a preferred peptide amphiphile, highlighting one or more structural features thereof. Region 1 may comprise a long alkyl tail that conveys hydrophobic character to the molecule and combined with the peptide region makes the molecule amphiphilic. Region 2 may comprise one or more (four consecutive, shown) cysteine residues which when oxidized may form disulfide bonds to polymerize the self-assembled structure. Region 3 may comprise a flexible linker region of one or more glycine residues, preferably three, or functionally similar such residues or monomers, to

provide the hydrophilic head group flexibility from the more rigid crosslinked region. Region 4 may comprise a single phosphorylated serine residue which is designed to interact strongly with calcium ions and help direct mineralization of hydroxyapatite. Region 5 may comprise a cell adhesion ligand RGD. b) Molecular model of an illustrated PA showing the overall conical shape of the molecule going from the narrow hydrophobic tail to the bulkier peptide region. c) Schematic showing the self-assembly of PA molecules into a cylindrical micelle.

Figure 2. a) Negative stain (phosphotungstic acid) TEM of self-assembled nanofibers before covalent capture. Fibers are arranged in ribbon-like parallel arrays. b) Vitreous ice cryo-TEM of the fibers reveals the diameter of the fibers in their native hydrated state to be 7.6 ± 1 nm. c) Positive stain (uranyl acetate) TEM of the self-assembled nanofibers after oxidative cross-linking showing electron dense regions due to the stain that localized on the periphery of the fibers. d) Thin section TEM of positively stained (uranyl acetate) nanofibers after oxidative cross-linking and embedding in epoxy resin. Two fibers are observed in cross-section (arrows) clearly showing the lack of staining in the interior of the fiber.

Figure 3. a) TEM micrographs of the unstained, cross-linked peptide-amphiphile fibers incubated for 10 min in CaCl_2 and Na_2HPO_4 solution. The fibers arranged in bundles are visible due to the high concentration of inorganic ions on their surface. b) After 20 minutes forming HA crystals (arrows) are observed in parallel arrays on some of the PA fibers. c) After 30 minutes mature HA crystals (arrows) completely cover the PA fibers. d) Electron diffraction pattern taken from a mineralized bundle of PA fibers after 30 minutes of exposure to calcium and phosphate. The presence and orientation of the diffraction arcs corresponding to the 002 and 004 planes indicate preferential alignment of the crystals with their *c*-axes along the long axis of the bundle. e) Plot of intensity versus inverse angstroms reveals that the 002 and 004 peaks of hydroxyapatite are strongly enhanced along the peptide-amphiphile fiber axis. f) EDS profile of mineral crystals after 30 minutes of incubation reveals a Ca/P ratio of 1.67 ± 0.08 as expected for HA.

Figure 4. Scheme showing possible relationships between peptide-amphiphile fibers and hydroxyapatite crystals in the mineralized bundle. Arrow indicates the direction of the *c*-axes of the crystals.

Figure 5. A tilt pair taken from mineralized PA fibers after 30 minutes of incubation with calcium and phosphate demonstrating the plate shape of the crystals. The crystals that were "edge-on" (electron dense, narrow objects) in the zero degree image lose contrast in the 45 degree rotated image (arrow 1) while the contrast of the crystals that were "face-on" in the zero degree images increase (arrow 2).

Figure 6. a) Nonphosphorylated PA fibers after 20 minutes of incubation with calcium and phosphate shows only amorphous mineral deposit concentrate on the fibers. b) Nonphosphorylated PA after 30 minutes of incubation with calcium and phosphate continue to show only amorphous mineral in contrast with phosphorylated PA which shows heavy crystallization at this time point.

Figures 7-9. TEM micrographs for several cylindrical micelles prepared from PA molecules listed in Table 1. Specifically: **Figure 7**, Top: Molecule #4 containing a C10 alkyl tail. Bottom: Molecule #13 containing a C22 alkyl tail; **Figure 8**, Top: Molecule 8 utilizing a tetra alanine sequence in place of tetra cysteine and containing a C16 alkyl tail. Bottom: Molecule 9 utilizing a tetra alanine sequence in place of tetra cysteine and containing a C10 alkyl tail; and **Figure 9**, peptide-amphiphiles with three different peptide head groups. Top: Molecule 10 with "KGE". Middle: Molecule 14 lacking the phosphoserine group. Bottom: Molecule 15 with "IKVAV".

Summary of the Invention.

In light of the foregoing, it is an object of the present invention to provide a nanostructured fiber-like system, or nanostructure providing other shapes such as spherical or oblate, and/or molecular components thereof, together with various methods for assembly and use to recreate or mimic the structural and/or functional interaction between collagen fibrils and hydroxyapatite crystals in bone or the extracellular matrix of bone prior to mineralization, thereby providing a nanostructured approach divergent from the prior art. It will be understood by those skilled in the art that one or more aspects of this invention can meet certain objectives,

while one or more other aspects can meet certain other objectives. Each objective may not apply equally, in all its respects, to every aspect of this invention. As such, the following objects can be viewed in the alternative with respect to any one aspect of this invention.

It is an object of the present invention to provide a composition which can be used for assembly of a molecular structure having dimensional and functional characteristics biomimetic with collagen fibrils.

It can also be an object of the present invention to provide a nanostructured fibrous system as a template for tissue development.

It can also be an object of the present invention to provide a composite of a mineralized nanofiber structure biomimetic with collagen fibrils and hydroxyapatite crystals in natural bone tissue.

It can also be an object of the present invention to provide a system for the facile self-assembly of nanostructured fibers for use in conjunction with one or more of the preceding objectives, such fibers as can be reversibly stabilized to promote structural integrity.

It can also be an object of the present invention to provide a methodology using fibers in a stabilized three-dimensional structure to direct and/or control mineralization and crystal growth thereon.

It can also be an object of the present invention to provide a molecular system for the design and engineering of specific nanofibers and components thereof to target particular cell and/or mineral growth en route to a variety of hard or soft biomimetic materials for biological and non-biological applications, the later including, but not limited to, catalysis, photonics and electronics.

Other objects, features, benefits and advantages of the present invention will be apparent from this summary and its descriptions of various preferred embodiments, and will be readily apparent to those skilled in the art having knowledge of natural biomineralizing systems. Such objects, features, benefits and advantages will be apparent from the above as taken into conjunction with the accompanying examples, data, figures and all reasonable inferences to be drawn therefrom.

With respect to various embodiments, the present invention comprises use of self-assembly techniques, such self-assembly as may be employed in conjunction with mineralization to prepare a nanostructured composite material which recreates or mimics the structural orientation and interaction between collagen and hydroxyapatite observed in bone. A composite may be prepared by self-assembly, covalent capture, and mineralization of one or more peptide-amphiphile (PA) compositions. As evident from the preceding, the PA compositions of this invention can be synthesized using preparatory techniques well-known to those skilled in the art -- preferably, by standard solid phase chemistry, with alkylation of the N-terminus of the peptide component. Mono or di-alkyl moieties attached to the N or C termini of peptides may influence their aggregation and secondary structure in water in both synthetic and natural systems. As illustrated in several embodiments, a hydrophobic, hydrocarbon and/or alkyl tail component with a sufficient number of carbon atoms coupled to an ionic peptide can be used to create an amphiphile that assembles in water into cylindrical micelles because of the amphiphile's overall conical shape. (J. N. Israelachvili *Intermolecular and surface forces*; 2nd ed.; Academic: London San Diego, 1992). The alkyl tails pack in the center of the micelle with the peptide segments exposed to an aqueous or hydrophilic environment. These cylindrical micelles can be viewed as fibers in which the chemistry of the peptide region is repetitively displayed on their surface. Comparably, consistent with this invention, amphiphile molecules can also be designed to provide micelles having structural shapes that may differ from a fiber like appearance.

Without limitation, three structural and/or functional features can be engineered into the peptide region of a PA composition of this invention. First, the prepared fibers are optimally robust and, for this reason, one or more consecutive cysteine amino acid residues -- four in some embodiments -- can be incorporated in the sequence for covalent capture of supramolecular nanofibers. (D. Y. Jackson, D. S. King, J. Chmielewski, S. Singh, P. G. Schultz, *J. Am. Chem. Soc.* **113**, 9391-9392 (1991); T. D. Clark, K. Kobayashi, M. R. Ghadiri, *Chem Eur J* **5**, 782-792 (1999); Y. Y. Won, H. T. Davis, F. S. Bates, *Science* **283**, 960-963 (1999); E. R. Zubarev, M. U.

Pralle, L. M. Li, S. I. Stupp, *Science* **283**, 523-526 (1999); E. A. Archer, N. T. Goldberg, V. Lynch, *J Am Chem Soc* **122**, 5006-5007 (2000); F. Cardullo, M. C. Calama, B. H. M. Snellink-Ruel, J. L. Weidmann, A. Bielejewska, R. Fokkens, N. M. M. Nibbering, P. Timmerman, D. N. Reinhoudt, *Chem. Comm.* **5**, 367-368 (2000)). Such residues can be used to form disulfide bonds between adjacent PA molecules upon oxidation to lock the supramolecular structure into place. The formation of the disulfide bonds is reversible allowing self correction of improper disulfide bonds or return to the supramolecular structure by treatment with mild reducing agents.

With regard to a second feature, the fibers of various embodiments may be able to nucleate the formation of HA crystals in the proper environment. It is well known that acidic moieties play a key role in biomineralization processes and in the formation of calcium phosphate minerals phosphorylated groups are particularly important. (G. K. Hunter, H. A. Goldberg, *Biochem. J.* **302**, 175-179 (1994); S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997)). For example in dentin, the phosphophoryn protein family contains numerous repeats of the sequences Asp-Ser(P)-Ser(P) and Ser(P)-Asp (A. George, L. Bannon, B. Sabsay, J. W. Dillon, J. Malone, A. Veis, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, *J. Biol. Chem.* **271**, 32869-32873 (1996)). These massively phosphorylated proteins are closely associated with the collagen extracellular matrix (ECM) and are known to play an important role in HA mineralization (A. Veis In *Biomineralization: Chemical and Biological Perspectives*; S. Mann, J. Webb, J. R. P. Williams, Eds.; VCH: Weinheim New York, 1989; pp 189-222). Accordingly, at least one phosphoserine residue can be incorporated into the peptide sequence which, after self assembly, allows the fiber to display a highly phosphorylated surface functionally biomimetic to a long peptide segment. This, in part, may be used to simulate a repetitive organization of phosphate groups found in phosphophoryn proteins.

Third, with respect to one or more of the preceding embodiments or others within the scope of this invention, it would be beneficial for biomedical applications to provide fibers promoting surface adhesion and growth of cells. Another collagen associated protein, fibronectin, contains the sequence Arg-Gly-Asp (RGD). As this

sequence has been found to play an important role in integrin-mediated cell adhesion, an RGD sequence can also be included in preferred peptide components and/or PA compositions, depending upon end-use application. Collectively, these and other design principles led to preparation of a PA molecule of the type shown in Fig. 1.

Notwithstanding the numerous embodiments provided above, broader aspects of the present invention include a peptide amphiphile composition having 1) a hydrophobic component and 2) a peptide or peptide-like component further including a cell adhesion sequence. In various preferred embodiments, the hydrophobic component of such a composition is of sufficient length to provide amphiphilic behavior and micelle formation in water or another polar solvent system. Typically, such a component may be about a C₆ or greater hydrocarbon moiety, although other hydrophobic, hydrocarbon and/or alkyl components could be used as would be well-known to those skilled in the art to provide similar structural or functional effect. Regardless, a peptide component of such a composition may include the aforementioned RGD sequence found especially useful for the nanofiber mineralization described herein.

Preferred peptide components of such compositions can also include a phosphoryl-functionalized (P) residue or sequence, as described above. Inclusion of a phosphoserine residue, S(P), has been found especially useful for HA mineralization. Other embodiments can include, for example and without limitation, a phosphotyrosine residue. The peptide component of such compositions can also include a residue or sequence capable of promoting intermolecular bonding and structural stability of the micelles/nanofibers available from such compositions. A sequence of cysteine residues can be used with good effect, providing for the facile intermolecular oxidation/reduction of the associated thiol functionalities.

Peptide components of this invention preferably comprise naturally-occurring amino acids. However, incorporation of known artificial amino acids and/or other similar monomers such as hydroxyacids are also contemplated, with the effect that the corresponding component is peptide-like in this respect. Accordingly, such artificial

amino acids, hydroxyacids or related monomers can be used to meet the spacer, phosphorylation and/or intermolecular bonding objectives described above.

Various aspects of the present invention can be described with reference to the peptide amphiphile illustrated in Figure 1, but consistent with broader aspects of this invention, other compositions can be prepared in accordance with this invention and used for the self-assembly of fibrous cylindrical micelles and corresponding nanostructures. See, Table 1, below.

Table 1

<u>PA</u>	<u>N-terminus</u>	<u>Peptide (N to C)</u>	<u>C-terminus</u>
1	C16	CCCCGGGS(P)RGD	H
2	C16	CCCCGGGS(P)	H
3	H	CCCCGGGS(P)RGD	H
4	C10	CCCCGGGS(P)RGD	H
5	C6	CCCCGGGS(P)RGD	H
6	C10	GGGS(P)RGD	H
7	C16	GGGS(P)RGD	H
8	C16	AAAAGGGS(P)RGD	H
9	C10	AAAAGGGS(P)RGD	H
10	C16	CCCCGGGS(P)KGE	H
11	C10	AAAAGGGS(P)KGE	H
12	C16	AAAAGGGS(P)KGE	H
13	C22	CCCCGGGS(P)RGD	H
14	C16	CCCCGGGSRGD	H
15	C16	CCCCGGGEIKVAV	H
16	C16	CCCCGGGS(P)RGDS	H
17	C _n	LLLKK-X	H
18	C _n	LSL-X	H
19	C _n	LSLS-X	H

It should be noted that within the particular system examined, PAs 3 and 5 do not exhibit micelle formation, illustrating a certain degree of hydrophobicity as may be useful in some embodiments for self-assembly of such compositions into the nanofibers or other nanostructures of this invention. Depending upon desired cell or mineral growth, a phosphorylated moiety or residue may not be included (see PAs 14 and 15). As discussed above, cellular adhesion or interaction may be promoted by a particular sequence of the peptide component. With reference to PAs 10-12 and 15, a non-RGD sequence can be utilized depending upon cellular target or end-use application. In particular, the IKVAV sequence has been identified in other contexts as important for neuron growth and development. The YIGSR sequence, known for a role in neuronal cell-substrate adhesion, can also be used. Accordingly, the amphiphile compositions of this invention can include a peptide component having such a sequence for corresponding use. Other residues and/or sequences capable of promoting cell adhesion, growth and/or development are known in the art and can be used in conjunction with the present invention, such residues/sequences as can be incorporated into the peptide components and/or PA compositions of this invention using available synthetic techniques or straight-forward modifications thereof. With respect to Table 1, it is noted that several PA compositions do not include cysteine residues: while such a residue or peptide sequence can be used to enhance intermolecular nanofiber stability, it is not required for micelle formation in the first instance. Reference is made to Figures 7-9 for TEM micrographs of several PA compositions identified in Table 1.

Further reference is made to Table 1 and, in particular, PA compositions 17-19. Various other embodiments of this invention may comprise the residues shown, or where optionally X may further comprise one or more of the aforementioned residues as may be utilized for intramolecular structural flexibility, intermolecular stability, mineralization and/or cellular interaction. Accordingly, each such composition can optionally comprise, as desired for end-use application, one or more glycine, cysteine, phosphorylated and/or cell growth, development or adhesion residues. In accordance with the preceding discussion, the amphiphilic nature of such compositions provides

for the presence of a suitably hydrophobic component C_n , where n is an integer corresponding to the number of carbon atoms in such a component sufficient to provide sufficient amphiphilic character and/or assembly structure. Without limitation, various embodiments of such compositions comprise a hydrophobic component of about C_6 - C_{26} . More specifically and without limitation, a sequence such as that provided by PA composition 17 (or as further comprising residue(s) X) can be utilized to modify and/or enhance the rate of nanostructure self-assembly. Likewise, without limitation, the sequences of PA compositions 18 and 19 (or as further comprising residue(s) X) can be used, as needed, to improve the structural or mechanical properties of the corresponding available nanostructured gel materials. Implementation of such peptides in the PA compositions of this invention do not, of course, preclude use of other residues. For example, PA compositions 17-19 can further comprise one or more glycine, cysteine, phosphorylated and/or cellular interaction residues, in accordance with this invention.

Various peptide-amphiphile compositions are shown in Table 1, but are provided only by way of illustrating one or more aspects of this invention. It will be understood by those skilled in the art that a range of other compositions are also contemplated. For example, the peptide components can be varied, limited only by functional considerations of the sort described herein. Accordingly, peptide length and/or sequence can be modified by variation in number or identity of amino acid or substituted monomer. Further, it will be understood that the C-terminus of any such sequence can be construed in light of associated carboxylate functionality or derivative thereof. While the N-terminus of the peptides is illustrated with respect to the referenced conjugated hydrophobic and/or hydrocarbon components, it will be understood that such components can also be varied by length and/or composition, such variation limited only by the functional considerations presented herein. For example, a sixteen carbon (C_{16}) component can comprise but is not limited to a straight-chain alkyl hydrocarbon. As would be understood by those skilled in the art, the structure and/or chemistry of such a component can be varied with regard to a particular, desired functionality of an amphiphile composition or assembly thereof.

Likewise, the length of such a component is limited only by way of the degree of hydrophobicity desired for a particular amphiphile composition and/or assembled structure, in conjunction with a given solvent medium.

In part, the present invention also provides a sol-gel system comprising 1) an aqueous or polar solvent solution and/or containing one or more of the amphiphile compositions described herein, and 2) a reagent to adjust system pH. As described elsewhere herein, a system pH of less than or equal to about 4, depending on peptide identity, induces assembly, gelation or an agglomeration of the solution with such compositions -- in some embodiments, biomimetic nanofibers of cylindrical micelles. Conversely, use of a suitable reagent to raise the system pH to greater than or equal to about 4 dissolves or disassociates the compositions or nanofiber micelles. In preferred embodiments, the amphiphile composition(s) of such a system includes a peptide component having residues capable of intermolecular cross-linking. The thiol moieties of cysteine residues can be used for intermolecular disulfide bond formation through introduction of a suitable oxidizing agent. Conversely, such bonds can be cleaved by a reducing agent introduced to the system.

Corresponding to one or more embodiments of such a material, composition or system, the present invention can also include a nanostructured template for mineral crystal and/or cellular growth. Such a template includes a micelle assembly of amphiphile composition(s) of the type described herein, wherein the peptide component thereof may include a residue or sequence capable of intermolecular bond formation. In preferred embodiments, as described above, cysteine residues can be used for intermolecular disulfide bond formation. Various other preferred embodiments can further include one or more phosphorylated residues to promote crystal growth and/or mineralization, depending upon a desired material or tissue target.

In the context of biomimetic hard material or tissue, the present invention can also include an organo-mineral composite having a nanostructured peptide amphiphile template with mineral crystals thereon. As described above, this aspect of the present invention can be illustrated with the present amphiphile compositions, assembled as

nanostructured fibers, used to nucleate and grow hydroxyapatite crystals. In preferred embodiments, the amphiphilic compositions include peptide components having one or more residues promoting crystal nucleation and growth. Such preferred peptide components can also include one or more residues capable of intermolecular bonding to stabilize the nanofiber template. While this inventive aspect has been described in conjunction with hydroxyapatite nucleation and growth, the mineral component of this composite can include other inorganic compounds and/or oxides. Such residues, sequences or moieties are of the type described herein, or as would otherwise be understood by those skilled in the art made aware of this invention.

Regardless, the c-axes of the mineral crystals of such composites are aligned with the longitudinal fiber axes, in a manner analogous to the alignment observed between collagen fibrils and HA crystals in natural bone tissue. Accordingly, the present invention can also include a method of using a peptide amphiphile, in accordance with this invention, to promote and control HA crystal growth. The identity of the PA compositions used therewith is limited only by way of those structural considerations described elsewhere herein. Consistent therewith and with the broader aspects of this invention, such a method includes 1) providing an aqueous or other suitable polar medium of one or more peptide amphiphile compositions, 2) inducing assembly thereof into cylindrical micelle structures, 3) optimally stabilizing the structures with intermolecular bond formation, and 4) introducing to the medium reagents suitable for the preparation of HA and crystalline growth thereof on the nanofiber micelle structures. As provided elsewhere herein, one or more amphiphile compositions can be used to provide fibers with a variety of cell adhesion, mineralization and/or structural capabilities. A combination of such compositions can be used to assemble a nanofibrous matrix with synergistic properties beneficial for a particular crystal and/or cellular development, such compositions as may vary according to peptide component, residue sequence, hydrophobic or hydrocarbon component and/or resulting PA or assembly configuration.

Examples of the Invention.

The following non-limiting examples and data illustrate various aspects and features relating to the compositions, micelles, composites and/or methods of the present invention, including self-assembly of a peptide-amphiphile nanofiber system, as is available through the methodologies described herein. In comparison with the prior art, the present structures, template designs and related methods provide results and data which are surprising, unexpected and contrary thereto. While the utility of this invention is illustrated through the use of several amphiphiles, biomimetic micelles and resulting organo-mineral composites, it will be understood by those skilled in the art that comparable results are obtainable with various other amphiphiles, nanofibers/micelles and/or composites, as are commensurate with the scope of this invention.

Example 1

After synthesis (see, example 10), the PA of Figure 1 (characterized by ^1H NMR and MALDI-TOF MS: $[\text{M-H}]^{-1} = 1333$) was treated with dithiothreitol (DTT) at a pH of 8 to reduce all cysteine residues to free thiols. At this pH the PA was found to be soluble in excess of 50mg/ml in water. However, upon acidification of the solution below pH 4 the material rapidly becomes insoluble. Solutions more concentrated than 2.5 mg/ml form birefringent gels in water that are self-supporting upon inversion of the container. Examination of the gels by cryo-TEM, which preserves the native, hydrated state of the material, revealed a network of fibers with a diameter of $7.6 \pm 1\text{nm}$ and lengths up to several microns (Fig. 2B). Negatives were digitized in the Umax PowerLook scanner with resolution 1200 dpi. The average thickness of the PA fibers was determined by two different procedures. First the Fourier transform of the images and following measurements of the distances between the peaks were performed in the NIH Image 1201.1262 program. The second approach applied was averaging technique based on the cross-correlation method, widely used in single particle reconstruction using the EMAN 1201.1202 program. For each data set 1200-1300 individual boxes were selected. (E. Beniash, W. Traub, A. Veis, S. Weiner, *J. Struct. Biol.* **132**, 212-225 (2000). Positively and negatively stained dried fibers were

found to have diameters of $6.0 \pm 1 \text{ nm}$ (Fig. 2A,C). TEM using the positive stain uranyl acetate, which preferentially stains acidic groups, revealed increased electron density at the periphery of the fiber. (J. R. Harris, Ed. *Electron Microscopy in Biology, A Practical Approach*; Oxford University Press: New York, 1991). Additionally, gels that were stained, embedded in epoxy resin and sectioned for TEM, showed fibers in cross-section in which donut shaped patterns were observed indicating staining only on the outer portion of the fiber (Fig. 2D). The two positive staining experiments of this example indicate that the hydrophobic alkyl tails pack on the inside of the fiber and the acidic moieties of the peptide are displayed on the surface of the fiber.

Example 2

The formation of the fibers was found to be concentration independent over more than three orders of magnitude (0.01 mg/ml to 50 mg/ml), however a second level of hierarchy was observed that was concentration dependent. As the concentration of the PA was increased, a larger number of the fibers were observed to pack into flat ribbons of fibers (Fig. 2A,C).

Example 3

Examination of the self-assembled material by FT-IR revealed a bimodal amide I peak with maxima at 1658 cm^{-1} (α -helix) and 1632 cm^{-1} (β -sheet) along with a N-H stretching peak at 3287 cm^{-1} indicating the formation of a hydrogen bonded structure, possibly utilizing a combination of β -sheet and α -helical secondary structure in the fibers (S. Krimm, J. Bandekar, *Adv. Protein Chem.* **38**, 181-364 (1986); W. K. Surewicz, H. H. Mantsch, D. Chapman, *Biochemistry* **32**, 389-394 (1993)). Based on the above data the nanofibers were modeled as cylindrical micelles in which the alkyl tails pack on the inside of the fiber and peptide segments are displayed on the outside containing both β -sheet and α -helical secondary structure. This model results in a fiber with a diameter of 8.5 nm , which is within the margin of error of our TEM measurements (Fig. 1C).

Example 4

Upon lowering the pH below 4 with HCl the material self-assembles; when the pH is brought back to neutrality with KOH it disassembles. This pH triggered self-

assembly and gelation allows the material to respond to its environment and thus may have applications in controlled release of molecules (W. A. Petka, J. L. Hardin, K. P. McGrath, D. Wirtz, D. A. Tirrell, *Science* **281**, 389-392 (1998)).

Example 5

Following self-assembly of PA molecules into fibers, the cysteine thiol groups were oxidized by treatment with 0.01M iodine. Examination of the material by TEM reveals that the fibers remain intact (Fig. 2C,D). These oxidized PA fibers were found to be stable to alkaline solutions (pH 8) for months while fibers which had not been oxidized would disassemble within minutes. Based on the length of the fibers revealed by TEM and the present model for molecular organization of fibers, the resulting polymer has a molecular weight of roughly 2×10^8 daltons. Importantly, the covalent capture process of this invention can be easily reversed by treating the fibers with a mild reducing agent such as DTT. After reduction the fibers regain their pH sensitivity and rapidly disassemble at pH 8. Together with the controlled self-assembly, reversible cross-linking provides a highly dynamic system which can interconvert between discrete molecules, self-assembled supramolecular fibers, covalently captured polymeric fibers and back again to single small molecules depending solely on the environment in which the material is placed.

Example 6

To investigate the mineralization properties of PA nanofibers of this invention, the material was assembled and mineralized directly on a holey carbon coated TEM grid -- to allow study of the dynamics of the mineralization process while minimizing artifacts of TEM sample preparation. To prepare samples, a drop of aqueous PA (1mg/ml) was mounted on the holey grid and the self-assembly of the PA was induced in an atmosphere of HCl vapor. The grids were immersed in aqueous iodine to oxidize the cysteine thiol groups to disulfides. After rinsing with distilled water, the PA coated grids were treated with 5 μ l of 10mM CaCl₂ on one side and 5 μ l of 5mM Na₂HPO₄ on the other side. The two solutions are able to mix only by passing through the holes in the carbon support. Grids were examined by TEM at different time intervals (Fig. 3) and after 10 minutes inorganic material was observed to be

concentrated around the fibers thus increasing their contrast (a crystalline phase was not detected at this time by electron diffraction). At 20 minutes the fibers begin to be covered with crystalline mineral, although significant amorphous material remains. After 30 minutes plate shaped polycrystalline mineral is visible throughout the surface of the fibers (Fig. 3C and Fig. 5). The mineral was analyzed by EDS (Energy Dispersion X-Ray Fluorescence Spectroscopy) which revealed a Ca/P ratio of 1.67 ± 0.08 which is consistent with the formation of HA with a formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (Fig. 3F).

Example 7

As controls for the experiment of example 6, carbon coated TEM grids were treated as above but without PA fibers. In this case no mineral deposit was found on the grids. In a second control, a PA was prepared in which phosphoserine was replaced by serine and treated as above with calcium and phosphate. The nonphosphorylated fibers were observed by TEM after 20 and 30 minutes of incubation and in both cases an amorphous mineral deposit around the fibers was observed, but crystals did not form within this time frame (Fig. 6).

Example 8

In order to discern the relative orientation of the HA crystals with respect to PA fibers, several samples of example 6, in which isolated mineralized bundles of PA fibers could be observed, were analyzed by electron diffraction. In all cases preferential alignment of the HA crystallographic *c*-axis with the PA fiber long axis was observed. (Fig. 3D,E).

Example 9

Mineralization experiments show that PA fibers of this invention are able to nucleate hydroxyapatite on their surfaces. Negatively charged surfaces can promote mineralization by establishing local ion supersaturation (S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997)). Particularly, the two acidic aminoacids phosphoserine and aspartic acid used here are abundant in the proteins of mineralized tissues proven to initiate crystal growth (L. Addadi, S. Weiner, *Proc. Natl. Acad. Sci.* **82**, 4110-4114 (1985); G. Falini, S. Albeck, S. Weiner, L. Addadi, *Science* **271**, 67-69

(1996); A. George, L. Bannon, B. Sabsay, J. W. Dillon, J. Malone, A. Veis, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, *J. Biol. Chem.* **271**, 32869-32873 (1996); S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997)). The fact that the fibers gain extra electron density prior to formation of the crystalline phase suggests the above mechanism may be utilized in our system. More surprising is the observation that the *c*-axes of the HA crystals are co-aligned with long axes of the fibers (Fig. 4). This fact implies that the orientation of crystalline nuclei and the subsequent crystal growth are not random but are controlled by the PA micelles. The exact mechanism of this control is not clear, however in similar systems such control is gained by specific arrangement of acidic groups that promote growth of the crystals in a particular orientation by an epitaxial mechanism (S. Mann, J. P. Hannington, R. J. P. Williams, *Nature* **324**, 565-567 (1986); S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997); J. Aizenberg, A. J. Black, G. M. Whitesides, *Nature* **398**, 495-498 (1999)). An analogous mechanism may be employed with PA fibers. Previous *in vitro* studies showing oriented crystal growth from organic templates were done mainly in two dimensional systems. The results of this example show for the first time oriented crystal growth in a synthetic fibrous organic substrate.

Example 10

Several representative peptide-amphiphiles of this invention were prepared by manual solid phase peptide synthesis starting from 0.5 mmoles of an Fmoc-Asp(tBu)-WANG resin. Deprotection of the initial and subsequent Fmoc groups and was accomplished by double treatment with 15ml of 30% piperidine in DMF for 2 minutes and 7 minutes. The resin was then washed with 15ml of DMF 5 times. With the exception of cysteine derivatives, amino acids (4 equivalents) were preactivated with HBTU (3.95 equivalents) and DiEA (6 equivalents) in 10ml of DMF for two minutes. The activated solution was then added to the deprotected resin and allowed to shake for 30 minutes after which a small sample was removed and analyzed with ninhydrin to test for completeness of the reaction. Failed reactions were recoupled in an identical fashion. Cysteine was coupled via the Fmoc-Cys(Trt)-OPfp activated ester with the addition of 1 equivalent of DiEA in order to avoid suspected

epimerization found using the standard Fmoc-Cys(ACM)-OH derivative with the conditions described above. Other amino acid derivatives used included Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Ser(PO(OBzl)OH)-OH. Analogous derivatives available in the art may be used to couple A, S, L, K and other such residues, including those shown in Table 1. Protecting groups may vary depending on the subject amino acid residue. The final step was coupling of a suitable fatty acid or derivative thereof (e.g., palmitic acid) and was accomplished using 2 equivalents of the acid activated with 2 equivalent of HBTU and 3 equivalents of DiEA in 15ml DMF for 2 minutes. The solution was allowed to react with the resin overnight. Likewise, other reagents and starting materials of the sort useful in the preparation of the inventive compositions, including but not limited to those shown in Table 1, are readily-available and known to those skilled in the art, such reagents/starting materials as may be used for the hydrophobic/hydrocarbon and peptide (residues/monomers) components.

Cleavage and deprotection of the peptide-amphiphiles was done with a mixture of TFA, water, triisopropyl silane (TIS) and ethane dithiol (EDT) in a ratio of 91:3:3:3 for three hours at room temperature. The cleavage mixture and TFA washings were filtered into a round bottom flask. The solution was roto-evaporated and then redissolved in a minimum of neat TFA. This solution was triturated with cold diethylether. The white precipitation was collected by filtration and dried under vacuum.

Various other amphiphile compositions of this invention can be prepared in analogous fashion, as would be known to those skilled in the art and aware thereof, using the above-described known procedures and synthetic techniques or straightforward modifications thereof depending upon a desired amphiphile composition or peptide sequence.

* * *

As demonstrated by the preceding examples, figures and data, the fact that the hydroxyapatite crystals grow on the bundles of fibers with their *c*-axes oriented along

the long axes of the micelles could be of interest for design of new materials for mineralized tissue repair. This nanoscale organization resembles that of hydroxyapatite crystals in mineralized ECM in which the HA crystals also grow in parallel arrays with their *c*-axes co-aligned with long axes of the organic fibers (W. Traub, S. Weiner, *Proc. Nat. Acad. Sci.* **86**, 9822-9826 (1989)). This arrangement is the most important characteristic of the biominerals belonging to the bone family (S. Weiner, H. D. Wagner, *Annu. Rev. Mater. Sci.* **28**, 271-298 (1998)). The organization of the collagen fibers, porosity, mineral-organic ratio vary in different members of this family, yet all of them are built from the collagen fibrils containing parallel arrays of hydroxyapatite crystals (W. Traub, S. Weiner, *Proc. Nat. Acad. Sci.* **86**, 9822-9826 (1989); S. Weiner, W. Traub, *FASEB J.* **6**, 879-885 (1992); W. J. Landis, K. J. Hodgens, J. Arena, M. J. Song, B. F. McEwen, *Microsc. Res. Techniq.* **33**, 192-202 (1996)).

While the principles of this invention have been described in connection with specific embodiments, it should be understood clearly that these descriptions are added only by way of example and are not intended to limit, in any way, the scope of this invention. For instance, various peptide amphiphiles have been described in conjunction with specific residues and corresponding cell adhesion, but other residues can be used herewith to promote a particular cell adhesion and tissue growth on the nanostructures prepared therefrom. Likewise, while the present invention has been described as applicable to biomimetic material or tissue engineering, it is also contemplated that gels or related systems of such peptide amphiphiles can be used as a delivery platform or carrier for cells or cellular material incorporated therein. Other advantages and features will become apparent from the claims filed hereafter, with the scope of such claims to be determined by their reasonable equivalents, as would be understood by those skilled in the art.

What is Claimed:

1. A peptide amphiphile composition comprising a peptide component with a cell adhesion sequence; and a hydrophobic component, said hydrophobic component sufficient to afford micelle formation of said composition in an aqueous medium.
2. The composition of claim 1 wherein said adhesion sequence comprises RGD.
3. The composition of claim 1 wherein said adhesion sequence comprises IKVAV.
4. The composition of claim 1 wherein said peptide component further includes at least one phosphorylated residue.
5. The composition of claim 4 wherein said phosphorylated residue is phosphoserine.
6. The composition of claim 1 wherein said peptide component further comprises a residue with a functional moiety capable of intermolecular covalent bond formation.
7. The composition of claim 6 wherein said residue is cysteine.
8. The composition of claim 7 wherein said peptide component further comprises at least one phosphoserine residue.
9. The composition of claim 8 wherein said peptide component further comprises at least one glycine residue between said cysteine and phosphoserine residues.
10. The composition of claim 8 wherein said cell adhesion sequence comprises RGD.
11. The composition of claim 10 wherein said hydrophobic component comprises an alkyl moiety of at least 6 carbon units.
12. The composition of claim 11 assembled into a cylindrical micelle.
13. The composition of claim 1 where in said peptide component is absent a cysteine residue.

14. The composition of claim 13 wherein said peptide component comprises an LLLKK sequence.

15. The composition of claim 13 wherein said peptide component comprises an LSL sequence.

16. The composition of claim 13 wherein said peptide component comprises an LSLS sequence.

17. The composition of claim 13 wherein said peptide component comprises an AAAAGGG sequence.

18. The composition of claim 13 wherein said cell adhesion sequence is selected from RGD, IKVAV and YIGSR.

19. The composition of claim 18 wherein said peptide component comprises at least one phosphorylated residue.

20. A sol-gel system comprising a solvent medium comprising a peptide amphiphile composition of claim 1, and a reagent to adjust system pH.

21. The system of claim 20 wherein said reagent is an acid, said pH is less than about 4, and said system comprises a gel of cylindrical micelles of said amphiphile composition.

22. The system of claim 21 wherein said reagent is a base, said pH is greater than about 4, and said system comprises a solution of said amphiphile composition.

23. The system of claim 20 wherein said peptide component of said amphiphile composition comprises a residue with a functional moiety capable of intermolecular covalent bond formation.

24. The system of claim 23 wherein said residue is cysteine.

25. The system of claim 24 further comprising an oxidizing agent to form a disulfide bond between amphiphile compositions.

26. The system of claim 25 further comprising a subsequent reducing agent to cleave said disulfide bond.

27. A nanofibrous micelle template for crystal growth, said template

comprising an amphiphile composition of claim 1, said peptide component of said composition comprising a residue with a functional moiety capable of intermolecular covalent bond formation.

28. The template of claim 27 comprising a plurality of cysteine residues, wherein the thiol moieties thereof are oxidized to form a disulfide bond between amphiphile compositions.

29. The template of claim 27 wherein said peptide component further comprises at least one phosphorylated residue.

30. The template of claim 29 wherein said phosphorylated residue is phosphoserine.

31. The template of claim 30 wherein said peptide adhesion sequence comprises RGD.

32. An organo-mineral composite comprising a nanofibrous micelle template of claim 30, and hydroxyapatite crystals on said micelle template, the c-axes of said crystals aligned substantially parallel with the longitudinal axis of said template.

33. A method of using a peptide amphiphile composition to control hydroxyapatite crystal growth, said method comprising:

providing a solvent medium with at least one peptide amphiphile composition, each said composition comprising a peptide component with a cell adhesion sequence and a hydrophobic component sufficient to afford micelle formation of said composition in said medium, said peptide component further comprising a phosphorylated residue and a cysteine residue;

inducing assembly of said peptide amphiphile compositions into cylindrical micelles;

stabilizing said micelles with intermolecular covalent bond formation;

and

introducing reagents for the preparation of hydroxyapatite.

34. The method of claim 33 wherein micelle assembly is induced by

adjusting the pH of said medium to less than about 4.

35. The method of claim 33 wherein micelles are stabilized by intermolecular oxidative coupling and disulfide bond formation.

1/9

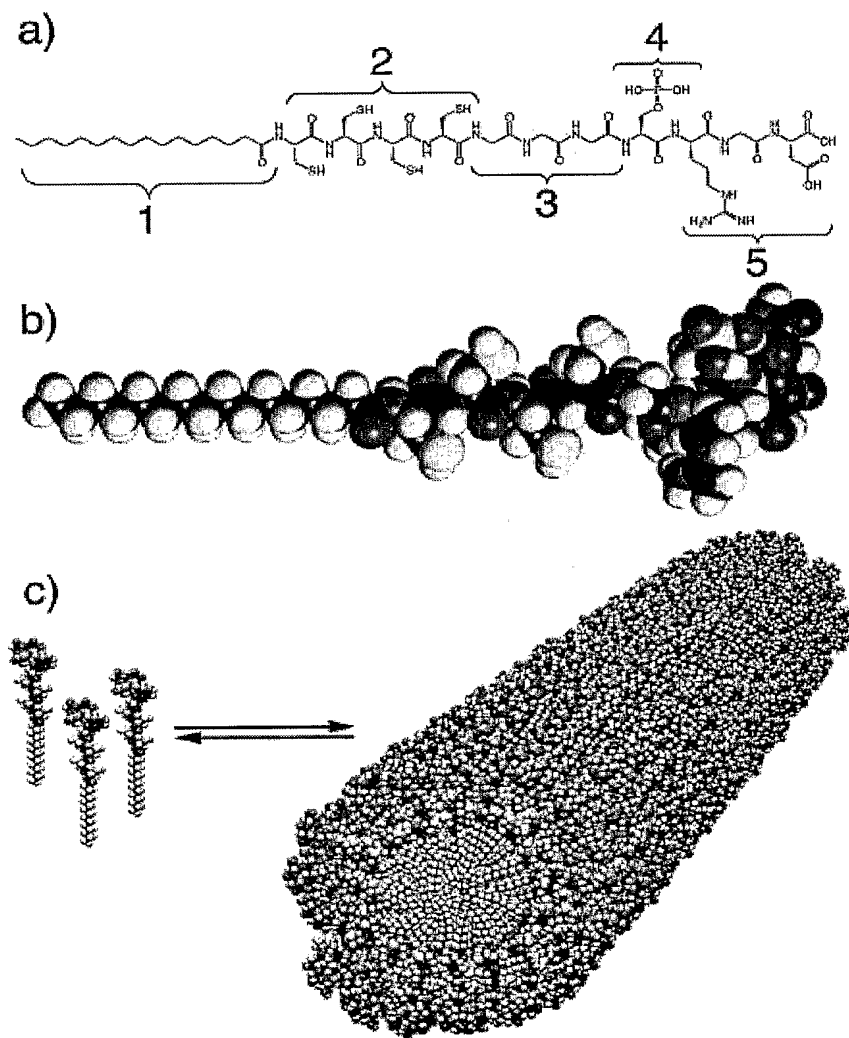


FIGURE 1

2/9

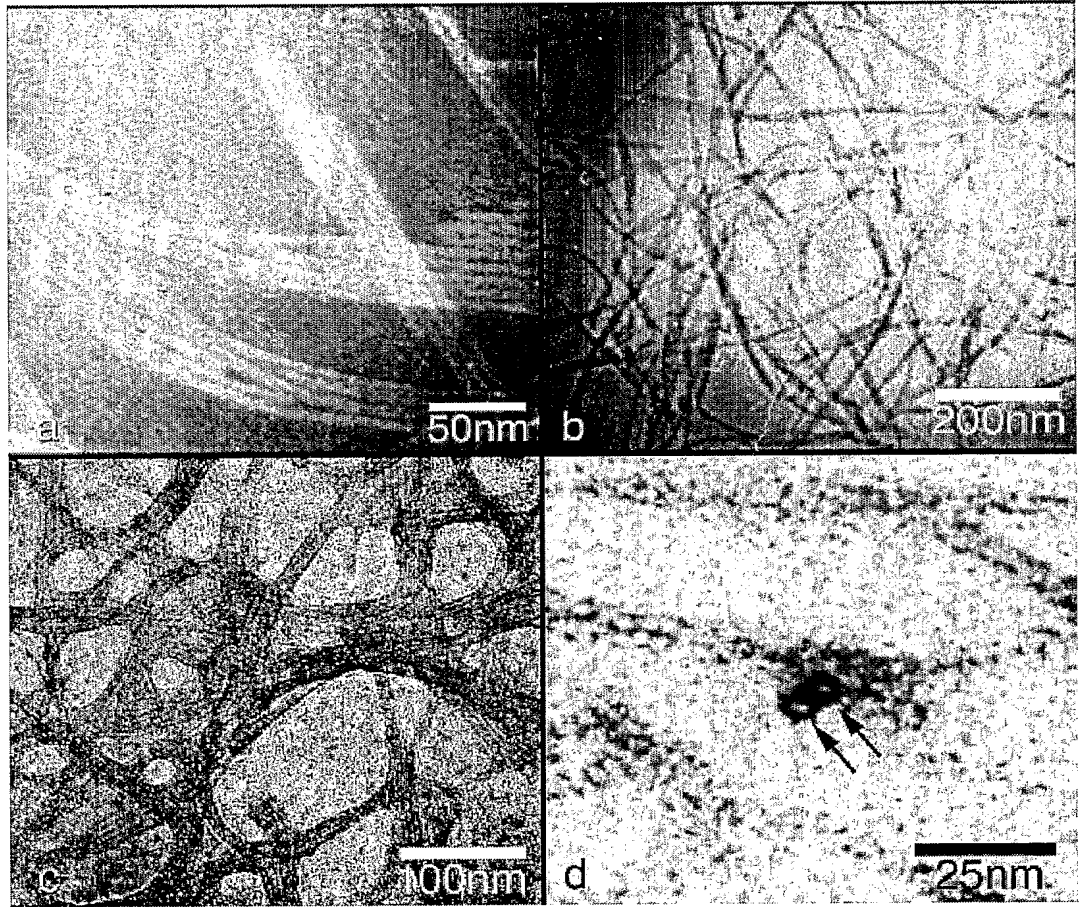


FIGURE 2

3/9

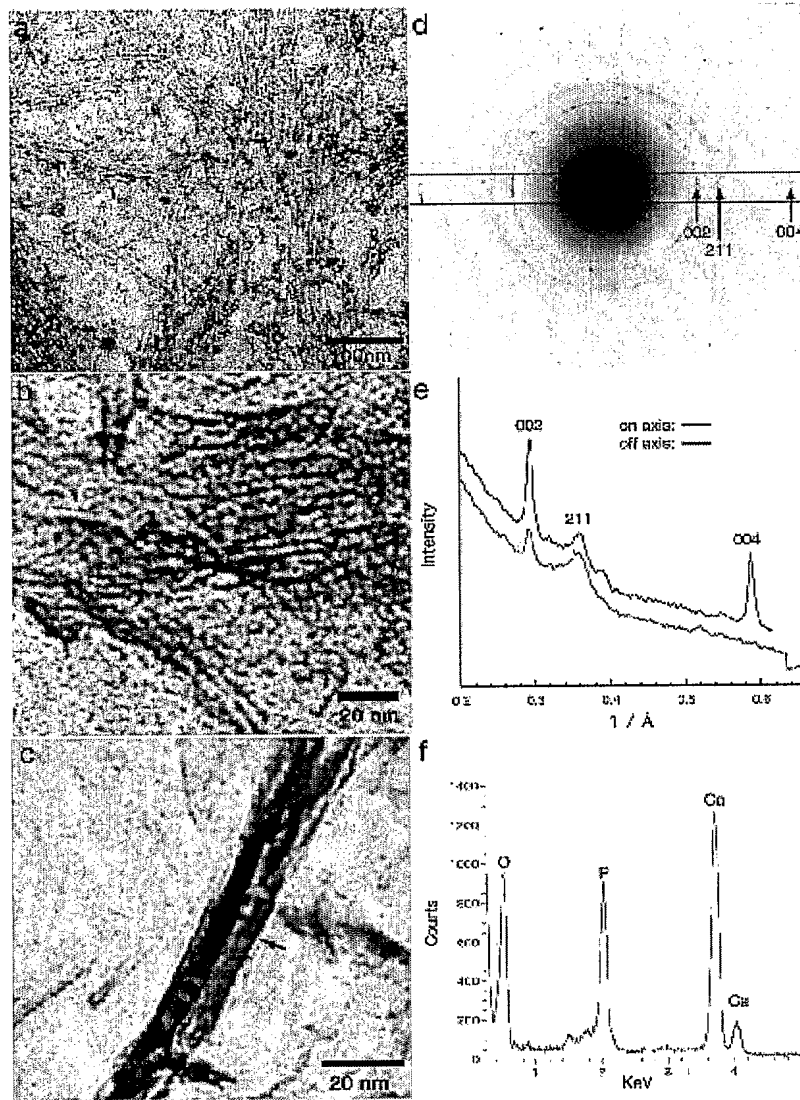


FIGURE 3

4/9

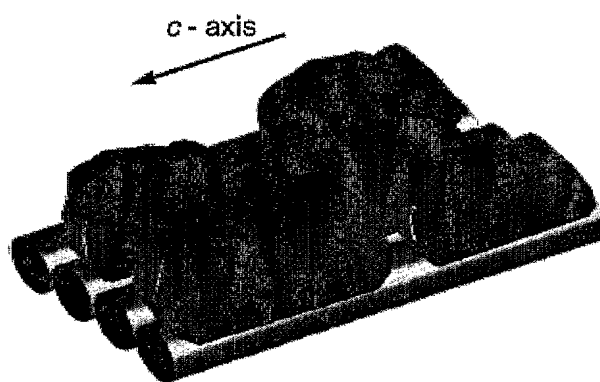
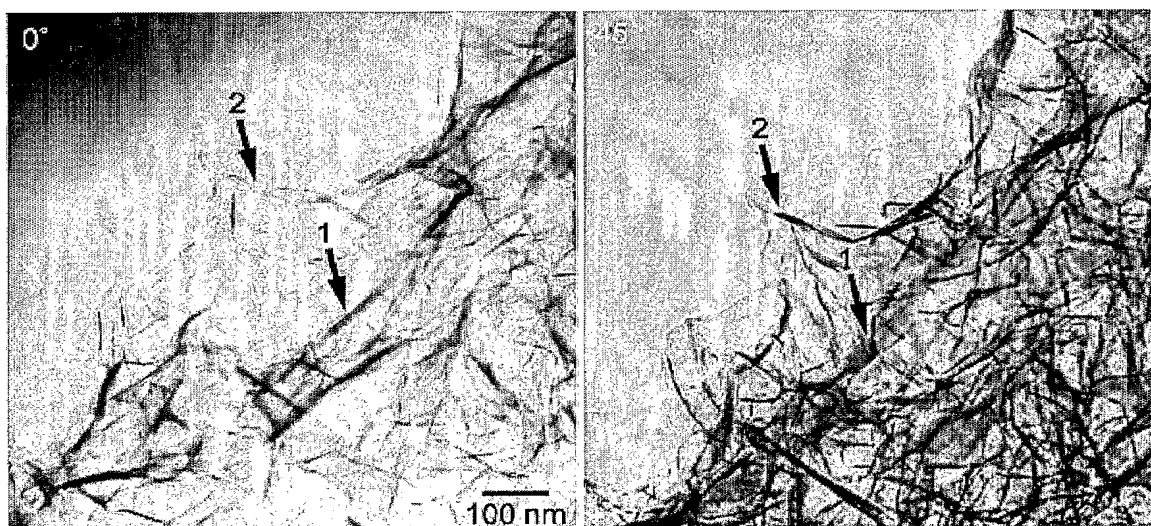


FIGURE 4

5/9

**FIGURE 5**

6/9

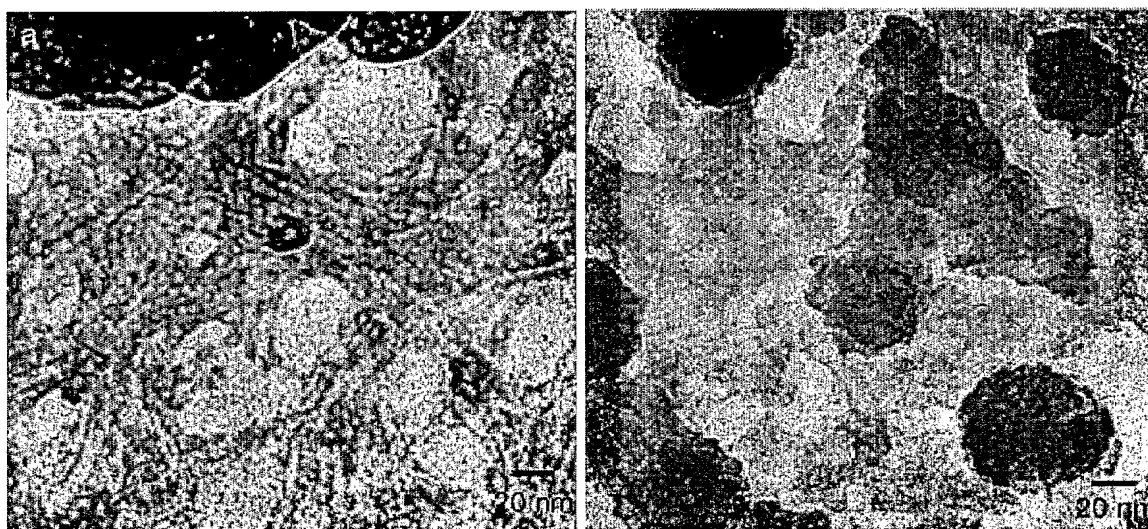


FIGURE 6

7/9

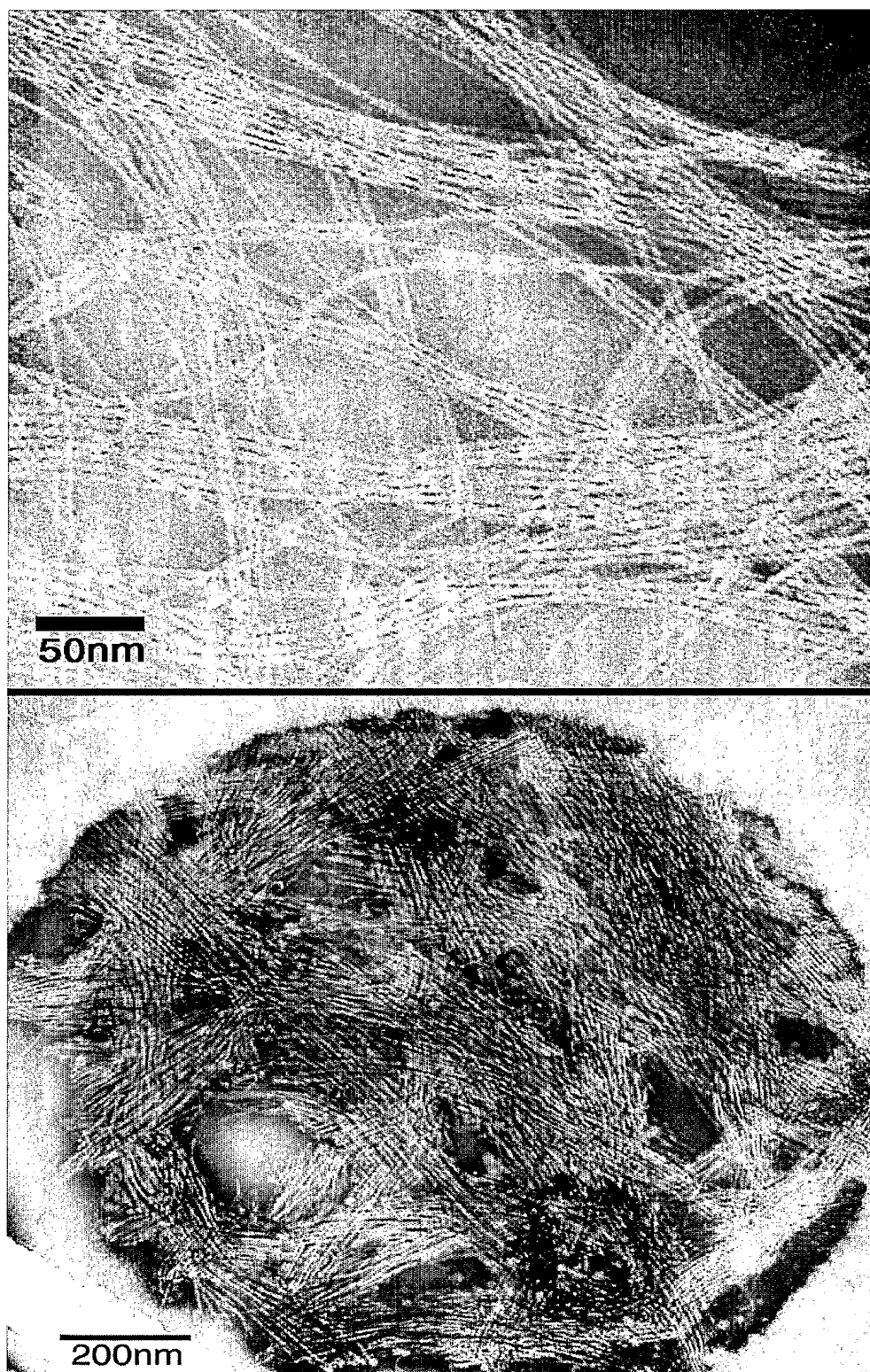


FIGURE 7

8/9

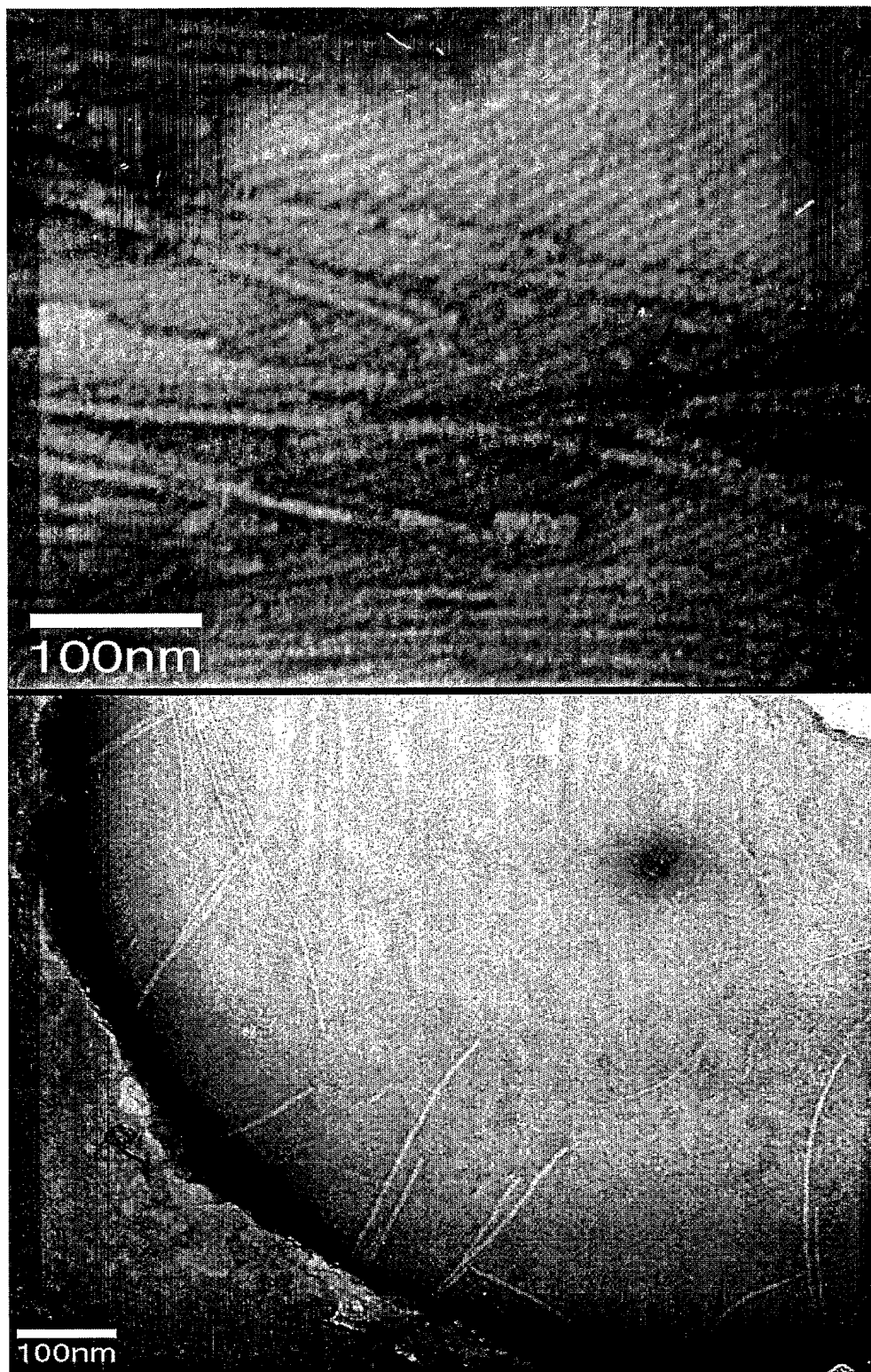


FIGURE 8

9/9



FIGURE 9